Journal of Medical Virology 56:332–336 (1998)

Saquinavir Delays the Emergence of Zidovudine Resistance in HIV-1 Seropositive Patients Treated With Combination Therapy

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During a randomized double-blind study to assess the antiviral activity of saquinavir (SQV) alone or in combination with zidovudine (ZDV), the emergence of phenotypic resistance was evaluated in 44 patients treated with SQV (13 subjects), ZDV (14 subjects), and SQV plus ZDV (17 subjects). A significant ($P < 0.05$) lower $CD4^+$ cell count and higher HIV RNA copy number at entry were found in six patients who developed resistant viral strain (3 to ZDV and 3 to SQV) during the first 4 months of treatment. After 1 year, drug-resistant strains (12 to ZDV and 14 to SQV) were isolated in 26 out of 37 patients. A significant higher number of patients treated with ZDV alone (10/13) harbored ZDV-resistant strains compared to patients treated by combination therapy (2/13); whereas more than 50% of patients had SQV-resistant strains aside from treatment. Early SQV-resistant strains were isolated in a limited number of patients treated with SQV alone (3/13). The rates of emergence of resistant strains during ZDV or SQV monotherapies are comparable. Combination therapy may delay the emergence of phenotypic resistance to either drugs in the short term and to ZDV, but not to SQV, at least after 1 year. ***J. Med. Virol. 56:332–336, 1998.***

KEY WORDS: combination therapy; HIV treatment; drug resistance; protease inhibitors

INTRODUCTION

Prolonged therapy with zidovudine (ZDV) is associated with the emergence of viral strains with decreased susceptibility to the drug in vitro [Mayers et al., 1992]. Some factors, such as a high error rate in the viral polymerase, a possible genetic recombination between genomes, and a strong in vivo selection, can contribute to the emergence of strains resistant to a nucleoside analogue. Furthermore, given the high replication rate of HIV and the frequency with which mutations occur during each replication cycle, it is not surprising that drug-resistant mutants of HIV appear during prolonged antiretroviral therapy.

The relationship between the development of resistance to individual antiretroviral agents and clinical outcome has not been fully established [Richman, 1994], even if high-level resistance of HIV to ZDV was associated with a decline in $CD4^+$ cell count [Kozal et al., 1993] and a more rapid clinical progression and death [D'Aquila, 1995]. The therapeutic effect of agents may be prolonged by combinations that suppress resistance to one another or antagonize mutations that are already present. Recent advances on drug resistance demonstrate that triple-combination therapies may reduce virus replication and delay significantly the emergence of drug-resistant mutants [Finzi et al., 1997; Wong et al., 1997]. Nevertheless, studies on the emergence of drug resistance during combination therapy show that simultaneous combination of ZDV plus ddC, ddI, or nevirapine do not delay the emergence of ZDV-resistant viral mutants [Richman et al., 1994a, 1994b; Holodniy et al., 1995].

Resistance to protease inhibitors was detected in vitro and in vivo. However, high doses of ritonavir and indinavir in monotherapy were effective in postponing the onset of resistance to drugs [Danner et al., 1995; Emini et al., 1996]. The combination of indinavir and ZDV resulted in a delay of drug resistance [Emini et al., 1996] and the synergistic antiretroviral activity of a combination of SQV plus ZDV and ddC was demonstrated in vivo [Collier et al., 1996].

Previously, in a randomized double-blind I/II trial

Contract grant sponsor: the Italian Ministry of Health, Istituto Superiore di Sanità, AIDS Research Projects 1996 and 1997.

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Accepted 17 June 1998

phase to assess the antiretroviral activity and tolerability of a HIV proteinase inhibitor (Saquinavir, SQV) alone or with ZDV, it was demonstrated that the combination therapy had the best in vivo antiviral activity in terms of plasma HIV RNA copy number and infectivity titer and higher and more sustained improvement in $CD4^+$ cell count [Vella et al., 1996].

Further data are presented on the phenotypic drug susceptibilities of HIV strains isolated from patients treated for 1 year with ZDV and SQV alone or in combination.

MATERIALS AND METHODS

Study Design

Forty-four patients with symptomatic HIV infection and $CD4^+$ lymphocyte count $\leqslant$300 cells/mm^3 who had not received antiretroviral treatment previously were enrolled in an evaluation of SQV efficacy. This was a 16-week, parallel, randomized double-blind study with blinded monthly extensions of therapy in the absence of major disease progression and toxicity [Vella et al., 1996]. The patients were treated three times daily with ZDV 200 mg (14 patients), SQV 600 mg (13 patients), and ZDV 200 mg plus SQV 600 mg (17 patients). All patients gave written informed consent and the study received official institutional and ethical approval.

Laboratory Monitoring

Heparinized blood samples for HIV isolation and EDTA-treated blood samples for HIV RNA quantitative PCR were obtained from patients at baseline, 1, 2, 4, and 12 months after the beginning of therapy.

RT-PCR assay (Roche Molecular Systems) to quantify HIV RNA copy number in plasma were carried out according to published techniques [Mulder et al., 1994]; viral load was measured after plasma dilution for HIV RNA copy number became higher than the cutoff value of the test.

HIV was isolated from plasma as described previously [Andreoni et al., 1992; Sarmati et al., 1994]. Briefly, 1 ml of polyethylene glycol (PEG) pretreated plasma sample was incubated in T25 flasks with 10×10^6 PHA-stimulated peripheral blood mononuclear cell (PBMC) obtained from seronegative donors. Cultures, placed in a humidified chamber at 37°C with 5% CO_2, were maintained for 40 days and monitored twice a week for p24 antigen production using a commercially available enzyme immunoassay (Abbott Laboratories, North Chicago, IL). A culture was considered positive if the concentrations of p24 exceeded 1,000 pg/ml in two consecutive determinations. Positive supernatants were harvested by centrifugation and stored in liquid nitrogen.

Sequential viral isolates derived from plasma culture were tested for drug sensitivity. Briefly, PHA-stimulated donor PBMCs (4×10^6 cells) were infected with 2 ml of medium contained viral stock adjusted to a multiplicity of infection of 2,000 $TCID_{50}$/ml. After a 2-hr adsorption period, aliquots of the cells washed twice in PBS were put into a 96-well plate containing five different concentrations of ZDV (0.001, 0.01, 0.1, 1, and 5 μM) or SQV (0.01, 0.1, 1, 10, and 100 nM). All culture assays were carried out in quadruplicate and monitored for p24 antigen production for 7 days after infection. Fifty percent inhibitory concentrations (IC_{50}) of drug against virus was determined based on comparative growth of isolates in untreated control cultures. HIV isolates were considered resistant to ZDV for IC_{50} >0.05 μM [McLeod et al., 1992] and resistant to SQV for IC_{50} >10 nM of SQV. All HIV strains isolated from the same patient were tested simultaneously for drug sensitivity.

To determine if the HIV isolates were syncytium-inducing (SI) or nonsyncytium-inducing (NSI), an aliquot of viral stock supernatant, containing 100 $TCID_{50}$, was cultured in T25 flasks with 10^6 MT-2 cells. Cultures were maintained for 4 weeks and were examined for syncytia twice a week. Syncytium formation was defined as at least 10 multinucleated giant cells in five high-power fields.

TABLE I. Phenotypic Resistance to Zidovudine and Saquinavir at Different Time of Treatment

	Saquinavir, number of resistant/tested		Zidovudine, number of resistant/tested	
Treatment	4 months	12 months	4 months	12 months
SQV + ZDV	0/17	7/13	0/17	2/13[a]
SQV	3/13	7/11	0/13	0/11
ZDV	0/14	0/13	3/14	10/13[a]

[a]$P < 0.01$.

Statistical Analysis

Statistical analysis of correlation coefficients was carried out using the Fisher's exact test (P two-tailed) and the Student's t-test was used for continuous measurements to test relationships in unpaired analysis. Group means were compared using analysis of variance. If the corresponding F-test was statistically significant then individual means were compared using the Bonferroni additive inequality, which checks for the maximum experimental error state (a).

RESULTS

At baseline, among the three groups of patients treated with SQV (13 subjects) and ZDV (14 subjects) alone or in combination (17 subjects), no significant difference in plasma HIV RNA copy number (log_{10} number of copies 5.9 ± 6.3, 5.6 ± 5.7, and 5.4 ± 5.6, respectively) and $CD4^+$ cell count (178 ± 97, 165 ± 88, and 189 ± 93, respectively) was detected.

Drug susceptibility of HIV isolates obtained from 44 and 37 participants completing, respectively, 4 and 12 months of study was examined. No reduction in drug susceptibility for SQV (mean IC_{50} 0.77 nM; range 0.09–2.4 nM) and for ZDV (mean IC_{50} 0.004 μM; range 0.001–0.019 μM) was detected for HIV isolates at baseline.

Data on phenotypic resistance to SQV and ZDV at different times of treatment are shown in Table I. After

TABLE II. Baseline Characteristics of Patients According to Drugs Sensitivity of HIV Strains Isolated During Antiretroviral Therapy

	Viral isolates		
	Sensitive	4 months resistant	12 months resistant
Number of patients	11	6	20
ZDV-resistant[a]	0	3	11
SQV-resistant[a]	0	3	9
SI phenotype (%)	3 (27)	3 (50)	13 (65)
CD4 cell/mm^3[b]	202 ± 85	87 ± 81[c]	191 ± 83
Log HIV RNA copy number/ml[b]	5.53 ± 5.64	6.15 ± 6.42[c]	5.51 ± 5.69

[a]Number of patients.
[b]Mean ± SD.
[c]$P < 0.05$.

4 months of treatment, resistant isolates were found in 6 out of 44 patients. In particular, ZDV-resistant isolates were obtained from 3 out of 14 patients treated with ZDV alone (mean IC_{50} 0.072 μM; range 0.05–0.09 μM) and SQV-resistant strains were isolated from 3 out of 13 patients treated with SQV alone (mean IC_{50} 23 nM; range 11–39 nM).

After 12 months of therapy, resistant strains were isolated from 26 out of 37 patients: 12 subjects showed a decreased sensitivity to ZDV (mean IC_{50} 0.27 μM; range 0.06–1.06 μM) and 14 to SQV (mean IC_{50} 29.4 nM; range 14–49 nM). A significant ($P < 0.01$) higher number (10/13) of patients treated with ZDV alone harbored ZDV-resistant strains compared to subjects treated with combination therapy (2/13). No significant difference in the emergence of SQV-resistant strains was found among the different arms of treatment. The SQV drug susceptibility of two ZDV-resistant strains among patients in combination therapy was 1.5 and 8.1 nM IC_{50}, respectively, and the IC_{50} mean of ZDV drug susceptibility of the seven SQV-resistant isolates was 0.019 μM (range 0.003–0.041 μM). HIV-resistant strain to either drugs was isolated from two patients only after 13 and 16 months of treatment (data not shown).

The baseline characteristics of patients according to the drugs sensitivity of HIV strains isolated during antiviral treatment are shown in Table II. Six patients with early emergence of resistant strain (after 4 months of treatment) had a significant ($P < 0.05$) lower $CD4^+$ cell count and higher HIV RNA copy number compared to patients with late emergence of resistant strain (after 12 months of therapy). No significant difference in $CD4^+$ cell count and HIV RNA copy number was found among patients with sensitive and late resistant isolates or in patients with ZDV- and SQV-resistant viral strains present at 12 months of therapy. Furthermore, no significant difference in the number of patients with syncytium-inducing viral strains was observed between the different groups of patients even if a higher percentage of SI phenotype was found in patients with resistant strains than in patients with sensitive strains (61% and 27%, respectively).

Changes in HIV RNA copy number and $CD4^+$ cell count are shown in Figure 1. Patients with early resistant strains showed a progressive decline of $CD4^+$ cell count and, after 1 year of treatment, they had a significant lower number of $CD4^+$ cells ($P < 0.05$) compared to patients with sensitive or late resistant strains.

A significant decrease in viral load (< 0.5 log) was observed in all patients after 2 months of therapy, but only patients with sensitive strain sustained a significant reduction of the viral load after 1 year of treatment.

After 2 years of therapy, no significant difference in $CD4^+$ cell number was detected between the two groups of 6 patients with sensitive HIV strains and in 13 patients with late resistant strains (9 to SQV and 4 to ZDV). Only patients with sensitive strains had a significant ($P < 0.05$) increase of $CD4^+$ cell count with respect to the baseline value (from 181 to 286 $CD4^+$ cells/mm^3) (data not shown).

DISCUSSION

Data are presented showing that a combination therapy with ZDV and SQV can result in the delay of development of resistance to ZDV and that after 12 months of therapy a significant improvement in $CD4^+$ cell count and decline in HIV viral load was detected only in patients with sensitive viral isolates.

Concomitant use of several antiretroviral agents may lead to additive or synergistic therapeutic effects acting preferentially on different cell types, and combination therapy may slow down the development of drug resistance if virus strains resistant to one drug are suppressed by the second drug. Moreover, it has been demonstrated that the suppression of viral replication, obtained with the highest drug doses, reduce the continued virus replication as well as the possibility of appearance of viral genome substitution associated with resistance [Emini et al., 1996].

The combination of ZDV plus SQV (1,800 mg/die) in antiretroviral naive patients may delay the emergence of phenotypic resistance to either drugs in the short term and to ZDV after 1 year at least. After 12 months of treatment HIV strains resistant to proteinase inhibitor were isolated in more than 50% of patients treated with SQV alone or in combination with ZDV. The failure of the combination therapy to delay the develop-

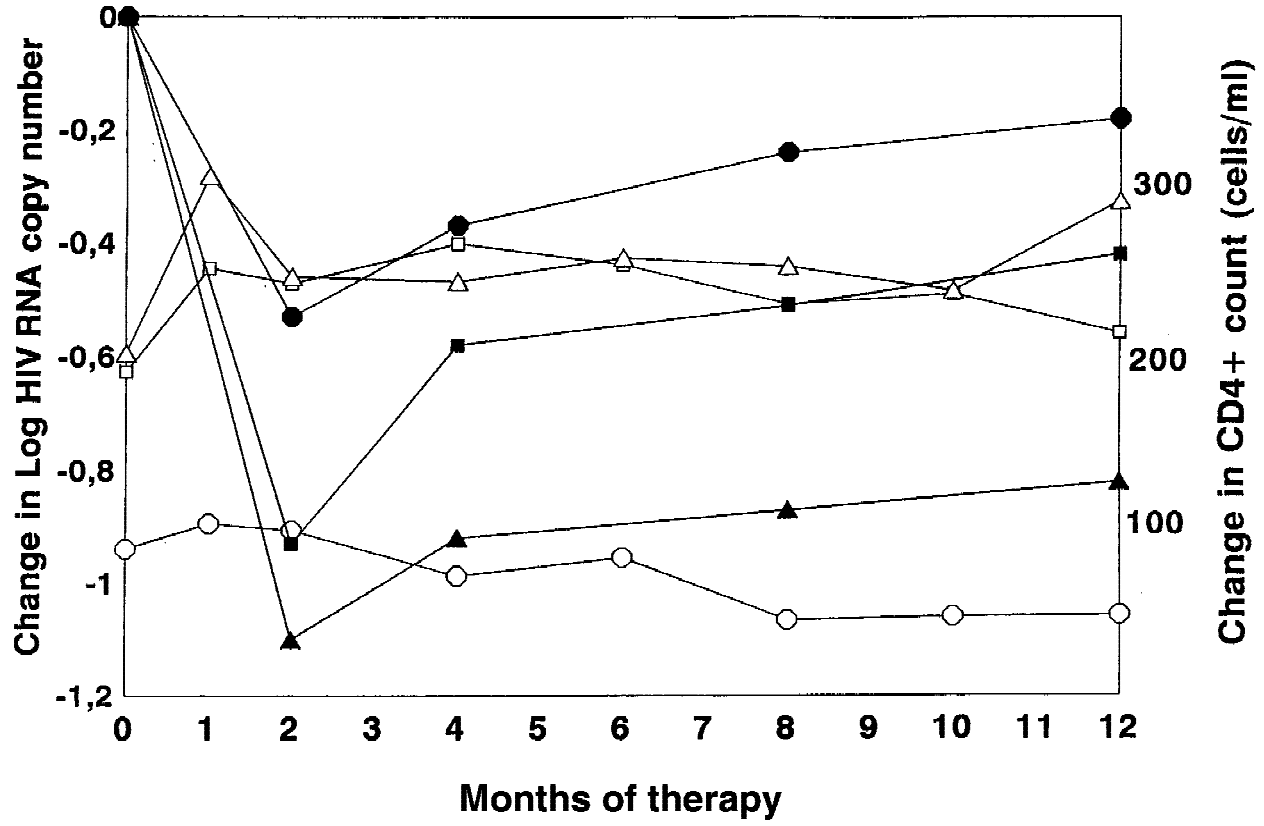

Fig. 1. $CD4^+$ cell count and log HIV RNA copy number in patients with HIV-sensitive isolates, early and late resistant to zidovudine or saquinavir during 1 year of antiretroviral treatment. $CD4^+$ cell count (△) and log HIV RNA copy number (▲) in patients with sensitive strains; $CD4^+$ cell count (□) and log HIV RNA copy number (■) in patients with early resistant strains; $CD4^+$ cell count (○) and log HIV RNA copy number (●) in patients with late resistant strains.

ment of SQV resistance could be explained by the characteristic low oral bioavailability of this drug. Outgrowth of SQV-resistant variant may arise from poorly penetrated compartments where viral replication persists in the presence of a selective but not virostatic level of the drug. Furthermore, all HIV proteinase inhibitors appear to be active against ZDV-resistant virus in vitro [Moyle, 1997].

An HIV strain was isolated resistant to both SQV and ZDV from two patients after 13 and 16 months of treatment, respectively. Thus, ZDV and SQV resistance are apparently not mutually exclusive, in agreement with their localization to different genes.

Before treatment, low $CD4^+$ cell count and high HIV RNA copy number were the strongest predictors of early emergence of resistance. Furthermore, the appearance of resistance during the first 4 months of treatment was found to be associated with a decline in $CD4^+$ cell count, while the isolation of HIV-sensitive strain after 1 year of therapy correlated with an increase of $CD4^+$ cell count sustained up to 2 years of therapy.

During the first 8 weeks of treatment, a decrease of HIV RNA copy number was observed in all patients apart from the development of resistant strain, even if patients with an early resistant isolate had the highest level in HIV RNA copy number every time. It is important to note that, after 1 year of treatment, only patients with a sensitive strain had a significant reduction in viral load, even if, after a initial rapid decline, viral level returns gradually to a steady state below baseline. This phenomenon has been described during antiretroviral therapy with nucleoside and nonnucleotide analogues and it is an effect of viral replicative dynamics [Havlir et al., 1996].

Resistant mutants to antiretroviral drugs are one of many quasispecies present in HIV-infected patients prior to treatment [Mohri et al., 1993; Nàjera et al., 1993]. On starting therapy, these drug-resistant mutants have a competitive advantage and become rapidly the dominant quasispecies [Frost et al., 1994; Kellam et al., 1994]. The lower number of replicative events occurring in patients treated with ZDV and higher dosage of SQV means that the time taken for an on-therapy equilibrium between quasispecies is reached after a longer interval than in patients with high viral burden. However, although proteinase inhibitors remain active against strains of HIV, which are resistant to reverse-transcriptase inhibitors, the long-term use of these agents as monotherapies may be limited by the development of resistance [Jacobsen et al., 1996].

In conclusion, HIV is a highly mutable virus that has revealed considerable in vivo variation. Mutants resis-

tant to most antiretroviral agents available at present have been reported and their genetic basis investigated. Combination therapy with ZDV and high dosage of SQV may delay the development of resistance to ZDV but not to SQV. However, further information is required about the frequency of different mutations during antiretroviral therapy, the pathogenicity of mutant strains, and the role of resistant mutants in clinical disease progression [Ercoli et al., 1997]. Long-term follow-up of the sensitivity of viral isolates during extended periods of time will be useful to clarify the overall picture of the emergence of resistance in response to combination therapy with SQV and ZDV.

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